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(54) HETEROCOMPLEXES OF INTERFERON WITH IMMUNOGLOBULIN AND PHARMACEUTICAL COMPOSITION THEREOF

(71) We, NG, MUN HON, of Flat 4, 142 Pokfulam Road, Hong Kong, and FUNG, KWOK PUI, of 26 Yan Oi Street, 1st Floor, San po King, Kowloon, Hong Kong, both of Chinese Nationality, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which is to be performed, to be particularly described in and by the following statement:-

The present invention relates to a pharmaceutical preparation containing interferon. It also relates to the active principle present in the preparation, and to methods of its preparation.

Interferon ('IF') is a potent and a general antiviral agent (see, for example, Ng, M. N., Vilcek, J.: Interferon: physicochemical properties and control of cellular synthesis. Adv. Protein Chem. 1972, *26*, 173-241) and may also possess anti-tumor activity (reviewed by I. Gresser in Anti-tumor effects of interferon. Adv. Cancer Res. 1972, *16* 97-140). Its therapeutic applications as such however have been limited up until now. This limitation is due in part to its pharmacokinetic properties since IFs have a relatively short serum half life. Native interferon, injected intramuscularly, typically has a serum half life of about 5 hours and a large dose is therefore necessary to achieve therapeutic effectiveness. It is difficult to purify IF and purification often leads to extensive reduction in the total activity (Knight, E.: Interferon: purification and initial characterization from human diploid cells. Proc. Nat. Acad. Sci. (U.S.A.) 1976, *73*, 520-523). This makes it impractical to consider the wide therapeutic use of pure IF at present. However, if IF was to be used before purification, the probability of adverse effects to the recipient due to the impurities may be expected to increase with the dosage administered.

In accordance with the present invention we now provide a heterocomplex comprising interferon cross-linked by a cross-linking agent to an immunoglobulin. Such hetercomplexes can be prepared by reacting excess immunoglobulin ('Ig') with interferon in the presence of a cross-linking agent. By so cross-linking the IF to the Ig the pharmacological properties of the IF are modified to the extent that effective IF therapy may be possible using smaller IF doses than have heretofore proved necessary.

Various cross-linking agents may be used and simple experimentation will determine whether a particular candidate for a cross-linking agent is indeed appropriate. By way of example, we mention that we have found that glutaraldehyde is a preferred cross-linking agent.

The IF-Ig hetercomplexes provided by the invention have different pharmacokinetic properties to IF itself, and yet exhibit biological properties of both the IF and Ig molecules. In addition, we have found some evidence for a possible adjuvant effect associated with the IF-Ig hetercomplexes.

The IF-Ig heterocomplexes can be obtained as soluble or as insoluble, gel-like products, in aqueous media depending on the particular conditions used for bringing about cross-linking. In particular, the extent of cross-linking can be controlled by the nature and amount of cross-linking agent used and by varying the pH of the reaction mixture. For instance, when using glutaraldehyde or similar agents, cross-linking probably involves reaction between the agent and the non-ionized form of the amino groups on the protein molecules; on this basis it is possible to rationalize the finding that a higher pH favours complex formation with such agents.

Biologically, the IF-Ig hetercomplexes are capable of inducing an antiviral state in diploid

human fibroblasts as does free native IF. The induction depends on an active states of synthesis of macromolecules by the cells. Unlike free IF, the heterocomplexes antigenically cross-react with Ig and may be precipitated by the antiserum against human Ig. The pharmacokinetics of the heterocomplexes are also different from the free IF. Although tissue retention at the injection sites and liver clearance of both the free and the complexed forms of IF appear similar, IF activity was not detected to the serum obtained from animals that received prior injection of the IF-Ig heterocomplex. This apparent discrepancy might be due to binding of the complexed IF onto blood cells. We have found that the heterocomplexed but not free forms of IF will bind with Raji cells which are a human lymphoblastoid cell line of B cell origin. As discussed below, this finding may be of use in quantitative assay of immune complexes.

From a further therapeutic standpoint, the heterocomplexes might have certain potential advantages over free IF. For example, blood-borneviruses in many instances are carried in the leukocytes. Thus the heterocomplexes, by virtue of their affinity for the leukocytes, might offer a means to control the dissemination of viral infection. In one embodiment an Ig having an antigenic specificity against a particular virus is used to prepare the heterocomplex; the specific Ig should then impart a "homing" property to the heterocomplex and thereby facilitate the therapeutic applications of IF.

In view of the expected therapeutic properties of the heterocomplexes, we also provide as part of this invention a pharmaceutical preparation which comprises a cross-linked IF-Ig heterocomplex, together with a pharmaceutically acceptable solid or liquid carrier. Liquid preparations can be injected into the patient, while solid preparations are suitable for topical application such as in the treatment of viral conjunctivitis.

Various aspects of the present invention will now become apparent from the following description and discussion of examples of the preparation and testing of IF-Ig heterocomplexes, using in this instance glutaraldehyde as the cross-linking agent. The drawings referred to are those which accompany the provisional specification, and percentages are by weight unless otherwise indicated.

Production and assay of interferon

Human fibroblast interferon was denoted by Dr. Jan Vilcek, New York University, New York, U.S.A. The method of superinduction as described by Havell and Vilcek (Production of high-titered interferon in cultures of human diploid cells. Antimicrob. Ag. Chemother. 1972, 2, 476-484) was applied in the preparation of IF from cultures of the human diploid fibroblast line, FS-4. The medium used for IF production contained 0.2% human plasma protein (final concentration). The resulting culture supernatant was precipitated with perchloric acid (0.25N) at 0°C and the precipitate containing IF activity was dissolved in 1/10 original volume of 0.02M phosphate, pH 7.4. The solution was then made to 40% by volume saturation with ammonium sulfate and the resulting supernatant was dialyzed against phosphate buffered saline ((PBS').

IF activity was assayed in microplate cultures of human diploid fibroblast using vesicular stomatitis virus as the challenging virus, according to Havell and Vilcek. The IF activity is expressed in actual "laboratory" units, 1 laboratory unit being determined as equivalent to 13/50 NIH standard units. The original crude IF which had contained 13,000 NIH standard units/ml, titered 50,000 laboratory units/ml in our assay method.

Preparation of immunoglobulin

An immunoglobulin can be prepared by precipitation of human sera at 40% by volume saturation with $(NH_4)_2SO_4$ followed by solubilization and equilibration against a phosphate buffer (0.04M, pH 7.4) and then application to a DE 52 column equilibrated with the same buffer. The fraction which is not absorbed on the column predominantly contains the immunoglobulin. Equally satisfactory results can be achieved using the immunoglobulin preparation available from Sigma Co., U.S.A.

The immunoglobulin was used in portions of 15.5 mg in one ml.

Reaction with glutaraldehyde

One ml aliquots of a solution containing 15.5 mg of human Ig and 1.5 mg protein of an IF preparation containing 2×10^4 IF units were dialyzed against acetate buffers containing 0.1M sodium acetate and 1M NaCl at pH values of 4.0 or 5.0 or against phosphate buffers containing 0.1M sodium phosphate and 1M NaCl at pH values of 6.0, 6.5, 7.0, 7.5 or 8.0. Different amounts of glutaraldehyde (Sigma Co., U.S.A.) were then added and allowed to react for 16 hours at 4°C. The reaction was stopped by the addition of 0.05 ml of 0.1M glycine of 1 ml of the mixtures followed by dialysis against PBS.

The IF-Ig heterocomplexes were then isolated from free IF by precipitation at 40% by volume saturation of $(NH_4)_2SO_2$, a condition which also caused the precipitation of Ig and

probably other Ig complexes as well.

The reaction conditions and the solubility of the product are shown in the following Table 1, in which a soluble product is indicated +, a partially soluble product ±, and an insoluble product −.

TABLE 1

pH	Glutaraldehyde : Protein*							
	500:1	50:1	40:1	30:1	20:1	10:1	5:1	1:1
4.0	+	+	+	+	+	+	+	+
5.0	−	+	+	+	+	+	+	+
6.0	−	±	+	+	+	+	+	+
6.5	−	±	±	±	+	+	+	+
7.0	−	±	±	±	±	+	+	+
7.5	−	±	±	±	±	+	+	+
8.0	−	−	±	±	±	±	+	+

*Molar ratio, based on molecular weights of Ig as 1.7×10^5 and that of glutaraldehyde as 100.

Assuming that the specific activity of pure IF is 2×10^8 units per mg and that IF has a molecular weight of 2×10^4 (cf. E. Knight, op. cit.), the molar ratio Ig:IF can be calculated as

$$\frac{15.5}{1.7 \times 10^5} : \frac{2 \times 10^4 \times 13/50}{2 \times 10^8 \times 2 \times 10^4}, \quad \sim 10^5:1$$

It can be seen from Table 1 that high pH and high concentration of glutaraldehyde relative to that of total protein in these mixtures favours extensive cross-linking of the protein molecules resulting in gelling of the solution (Table 1). Once gelled, the complexes thus formed have molecular weights in excess of 10^6 which could not be electrophoresed into acrylamide gel slab. At a glutaraldehyde to protein molar ratio of 5 at pH 7.5 or 8.0, however, soluble complexes were obtained which could be subjected to electrophoresis.

Electrophoresis

Gel slabs were cast in a linear gradient of acrylamide of 4 to 24% made up in 0.1M phosphate buffer, pH 7.2 containing 0.1% sodium dodecyl sulfate (SDS). Electrophoresis was performed at 6 volts per slab for 40 hours and the slabs were then either stained with "Coomassie" (Registered Trade Mark) blue or sliced. The gel slices were eluted by shaking in 0.5 ml of PBS at 4°C for 24 hours and IF activity thus eluted was assayed.

In Figure 1 there are shown the electrophoretic patterns obtained with, reading from the left; A: protein marker mixture containing thyroglobulin (Thy), rabbit IgG (IgG), bovine serum albumin (BSA), α-chymotrypsinogen (α-Chym), and myoglobulin (Myo); B: native IF preparation; C: IF-Ig mixture after reaction at pH 7.5 with glutaraldehyde at a molar ratio to protein of 5:1; D: same IF-Ig mixture as C except reaction occurred at pH 8; E: same IF-Ig mixture as C before reaction with glutaraldehyde. The soluble heterocomplexes obtained using the process of the invention migrate as large molecular weight bands (Figure 1 C & D) that are absent in the stained electrophoretic patterns of the IF-Ig mixture before reaction (Figure E) or in that of the native IF preparation (Figure 1B).

The amount of IF present in the IF-Ig mixtures was too small to allow its resolution as stained bands. Accordingly, gel slabs which had been electrophoresed under similar conditions as described for Figures 1B and 1D, but without fixing or staining, were sliced. The gel slices were eluted with PBS and the electrophoretic migration of IF activity was assayed. Figure 2 shows the results obtained; Figure 2*a* shows the migration pattern for the native IF preparation and Figure 2*b* shows the migration pattern for the reacted IF-Ig mixture. Also included in Figures 2*a* and *b* is the calibration curve derived from the protein

marker mixture as described for Figure 1A. It will be seen from Figure 2*a* that native IF migrates as a species with a molecular weight of 20,000. This result is in accord with the findings of other workers (see E. Knight, *op.cit* and Vilcek, J., Havell, E.A., Yamazaki, S. : Antigenic, physiochemical and biological characterization of human interferons. Ann. N.Y. Acad. Sci. (in press)). On the other hand, Figure 2*b* shows that the complexed IF activity migrated as a heterogeneous species with molecular weight ranging between 0.5 to 1.0 x 10^6 dalton. The 20,000 molecular weight species was conspicuously missing from the latter migration pattern.

It is apparent that although IF in the native or complexed forms could not be detected as stained bands, the reaction with glutaraldehyde had obviously covalently linked the IF molecules giving rise to the large molecular weight species with IF activity as observed in Figure 2*b*. The electrophoretic conditions employed (i.e. prolonged electrophoresis (40 hours) and the presence of SDS) probably exclude the possibility that these large molecular weight species are due to IF molecules which are non-covalently associated with other molecules. Moreover, bearing in mind the large excess of Ig relative to IF in the reaction mixture, it seems most likely that the large molecular weight species are hybrid molecules containing both IF and Ig.

Further characterisation of IF-Ig complexes

The proposition that the large molecular weight species are indeed heterocomplexes of Ig and IF is confirmed by the results of the following experiments.

Firstly, as a duplicated experiment, the reaction mixtures before and after treatment with glutaraldehyde at pH 8.0 were taken to 40% by volume saturation of $(NH_4)_2SO_4$. The distribution of IF activity in the precipitated and supernatant fractions is shown in Table 2.

TABLE 2

Fraction		IF activity (Units) Experiment 1		Experiment 2	
		Test	Control	Test	Control
Starting material		27,000	27,000	40,000	27,000
40% $(NH_4)_2SO_4$	precipitate	14,000	126	27,000	125
	Supernatant	25	18,000	1,250	12,000

It is apparent that whereas the native IF is not precipitable, the complexed IF activity is precipitated at 40% by volume $(NH_4)_2SO_4$ (as also are immunoglobulins). The increase in the recovery of IF activity observed in Experiment 2 after reaction with glutaraldehyde is probably insignificant in that the difference is within the error of IF assay.

In a second set of experiments, an anti-Ig test was performed using two preparations of the IF-Ig heterocomplex and one of native IF. The preparations were allowed to react with anti-human immunoglobulin, first at room temperature for 2 hours and then at 4°C overnight. The complexed IF was found to be precipitable which was presumably due to reaction of Ig in the hybrid molecules with its corresponding antibodies. By contrast, the native IF remained soluble. The results are tabulated in Table 3.

TABLE 3

	Interferon Titres		
	IF-Ig Hetero-complex (1)	IF-Ig Hetero-complex (2)	Native IF
Control	14,000	27,000	27,000
Supernatant	15	2,430	24,300

The antiviral activity of IF is not exerted directly at the virus or its replication. Rather, this activity is directed primarily at the cells i.e. IF treatment of cells renders the cells refractile to infection with viruses (Ng & Vilcek *op cit*). The development of this refractile state, commonly referred to as the antiviral state, occurs following treatment of cells with IF and as such requires active cellular macro-molecular synthesis.

The effect of actinomycin-D on the induction of the antiviral state by the IF-Ig complexes was studied. Actinomycin D is an agent which arrests cellular RNA synthesis. Cultures of human fibroblasts were treated or not with 0.25 µg per ml of actinomycin-D for 60 minutes. They were then washed free of the drug before exposure to 0, 2, 20 or 200 IF units of the complexed IF-Ig preparations or native IF, for 12 hours. The cultures were washed and challenged with vesicular stomatitis virus (VSV) at a multiplicity of 0.1. The culture supernatant was assayed after 24 and 72 hours for the yield of VSV by plaque assay using primary cultures of chick embryo cells. The treatments and tests results are shown in Table 4.

TABLE 4

Test	Treatment of Culture														
	Actinomycin-D							None							
Results	Complexed IF (units)				Native IF (units)			Complexed IF (units)				Native IF (units)			
	0	2	20	200	2	20	200	0	2	20	200	2	20	200	
CPE (48 hrs)	+	+	+	+	+	+	+	+	+	–	–	+	–	–	
24 hrs. Virus Yield (PFU x 10^{-3} per ml)	13	30	2	2	8	2	16	14	2	<0.1	<0.1	2	0.2	<0.1	
72 hrs. Virus Yield (PFU x 10^{-5} per ml)	4	7	0.6	2	5	0.9	1	4	4	<0.01	<0.01	2	<0.01	<0.01	

As is shown in Table 4, pretreatment of the cells with actinomycin D inhibits the development of the IF-mediated antiviral state i.e. IF treatment did not afford protection against subsequent infection with VSV to cells pretreated with actinomycin D. Thus, despite treatment with a protective dose of IF, these cells developed a cytopathic effect (CPE) as did the control cells that had not been exposed to IF, when challenged with the virus. These treated cells also supported virus replication to the same extent as the control cells, as is evident from the fact that both yielded similar plaque forming units (PFU) of VSV.

The antiviral effect of the complexed IF is also mediated via the cells in a similar manner to native IF. This effect is absent when the cells are pretreated with actinomycin D. It may be concluded from these results that the complexed IF retained the antiviral activity of the native IF. It may also be concluded that the hybrid or hetero molecules of Ig and IF also have in addition the physicochemical and biological properties of Ig, in that the molecules are precipitable by antiserum against Ig and at 40% by volume saturation of $(NH_4)_2SO_4$ (like native Ig's).

As will now be explained, those properties which are thus far known to be unique to the hybrid or hetero molecules include (1) pharmacokinetic properties and (2) affinity of the complexed IF for Fc receptors.

Preliminary pharmacokinetic studies of IF-Ig heterocomplexes

The pharmacokinetic properties of the complexed IF were studied by intramuscular administration of like doses of the native and complexed IF to Swiss mice. Randomly bred male Swiss mice weighing approximately 35 g were given intramuscular injection of IF or of IF-Ig heterocomplexes in amount of 0.05 to 0.1 ml of PBS containing approximately 10,000 IF units. The animals were bled from the tails at different times and IF activity in the serum specimens were assayed.

The results are shown in Figure 3.

The serum IF levels in mice receiving the native IF reached an early maximum at or before 30 minutes following injection, and then declined rapidly. The serum half-life of native IF in the mice was estimated to be about 3 to 5 hours. On the other hand, mice which were given the same dose of the complexed IF did not shown a detectable serum IF activity for up to 4 days following injection.

A test was carried out to determine if the above-described difference was due to tissue retention at the sites of injection or to a more rapid liver clearance of the complexed IF. Mice were given an intramuscular injection of 10,000 units of native or complexed IF, bled 4 hours later, and sacrificed. Tissues at the sites of injection and the livers were excised, minced and extracted for 24 hours with phosphate buffer (3ml of 0.02M phosphate buffer pH 7.4) containing 0.1% SDS to 1 gram of tissue. The extracts were clarified by centrifugation at 12,000 g for 30 minutes. The sera and tissue extracts were then tested for IF activity and the results are shown in Table 5.

TABLE 5

Animal No.	Form of IF administered	IF Activity		
		Muscle (units per g tissue)	Serum (units per ml)	Liver (units per g tissue)
1	Native	450	60	180
2	Native	300	65	270
3	Complexed	450	<45	180
4	Complexed	450	<45	180
5	None	<50	<45	<15

It is apparent from Table 5 that, as indicated by the levels of tissue IF the turnover of the native and complexed IF in the mice is similar. Serum IF in mice receiving the complexed IF was again not detectable. The blood cells from these animals were extracted but the level of blook cell-bound IF activity was probably too low and could not be detected.

Fc receptor binding

As an alternative approach, binding with human lymphoid cells *in vitro* was used as an experimental model. 5×10^6 Raji cells were suspended in 0.5 ml of medium or medium containing 2.5 mg of an ovalbumin-antiovalbumin complex (ova) or 0.5 mg of heat-inactivated human IgG (ΔIgG). The cells were allowed to stand for 30 minutes at room temperature and 0.5 ml of complexed IF was then added. The mixture was allowed to stand for 30 minutes at room temperature, centrifuged, and the supernatants were assayed for IF activity. Complexed IF mixed with equal volumes of the medium or the medium containing the same amount of ova of ΔIgG served as controls.

The results are shown in Table 6.

TABLE 6

Reaction mixtures	IF titers (units per ml)
Cells + complexed IF	<5
Cells + ova + complexed IF	45
ova + complexed IF	45
complexed IF	45
Cells + complexed IF	<3
Cells + IgG + complexed IF	18
IgG + complexed IF	18
complexed IF	18

It can be seen that the complexed IF binds with Raji cells, a human lymphoid cell line that contains Fc receptors. This result is in contradistinction to that obtained with native IF. Binding of the IF-Ig was inhibited when the Raji cells were first incubated with the soluble ovalbumin-antiovalbumin immune complexes or with human IgG that had previously been subjected to heating at 63°C for 30 minutes (ΔIgG). The extent of inhibition was found to depend on the dose of the soluble immune complex or ΔIgG. These results are consistent with the hypothesis that binding of the complexed Ig-IF is due to interaction between the Ig moiety on the hybrid Ig-IF molecules and the Fc receptor on the Raji cells.

The acquisition of an affinity for Fc receptors in a unique property of the complexed Ig-IF, and is believed to be due to denaturation of the Ig moiety during the process of cross-linking. Whatever the underlying reasons, this finding has two implications; (1) it may account for the failure in detecting serum IF activity in mice receiving injection of the complexed IF; and (2) it seem possible to quantitatively analyse for soluble immune complexes by their interference with binding of the complexed IF with Raji cells.

If established, the latter implication would have definite advantages over the current radioactive assay using ^{125}I labbeled ΔIgG for assay of immune complexes. The sensitivity of the complexed IF binding assay is potentially as sensitive as the radioassay, being capable of detecting 500 ug or less of the ΔIgG; however the complex IF has a more stable shelf life (it is stable for at least four months while the half life of ^{125}I ΔIgG is that of the radioactive isotope).

Further purification of IF·Ig heterocomplexes

The results above also point to a way to purify the complex IF. The preparative procedure included a first precipitation of the complex IF at 40% by volume $(NH_4)_2SO_4$. This step would have allowed the separation of non-immunoglobulins including native IF from the reaction mixture.

Further purification might be achieved by affinity chromatography of the precipitated fraction on an immobilised neuraminidase column, using the method described by Fung and

Ng (Fung, K.P., Ng, M.H. : Purification of human diploid fibroblast interferon by immobilised neutraminidase. Arch. Virol. (in press)).

This purification makes use of the affinity of the immobilised neuraminidase at pH 4.5 for molecules such as IF which contained sialic acid molecules. In a typical procedure, a preparation of human IF-Ig complex containing about 1000 units of IF per ml was obtained by allowing a mixture of IF and Ig to react with glutaraldehyde at a ratio to protein of 5 and at pH 8.0 followed by the precipitation at 40% by volume saturation of $(NH_4)_2SO_4$. One ml of this preparation was dialysed against acetate buffer pH 4.5 and applied to a column (0.9 x 16 cm) containing approximately 0.24 units of neuraminidase (Sigma Co., U.S.A.) conjugated to agarose beads such as those available under the name Sepharose 4B, "Sepharose" being a registered trade mark. The column was developed with 0.1M $NaHCO_3$ buffer pH 9.5.

As can be seen from the results of the chromatography plotted in Figure 4, the bound heterocomplexes are eluted at pH 9.5. At pH 9.5 there is negligible enzymic activity which therefore results in the dissociation of the substrate (i.e. IF-Ig) from the immobilised enzyme. A better than 20-fold purification of the IF-Ig can be achieved by a single cycle through the immobilised enzyme column, with a recovery of better than 70% of the total IF activity initially applied.

Other cross-linking agents

Although the foregoing examples are concerned with IF-Ig heterocomplexes obtained using glutaraldehyde as the cross-linking agent, it will be apparent that other cross-linking agents suggest themselves as candidates for use in the preparation of the heterocomplexes. Experimental evaluation will show whether a potential agent is indeed suitable. Regardless of the agent employed, the resultant heterocomplex will have pharmacokinetic and other properties which are different to those of IF and Ig themselves and which will lend the heterocomplex to various biological uses.

WHAT WE CLAIM IS:-

1. A heterocomplex comprising interferon cross-linked by a cross-linking agent to an immunoglobulin.
2. A heterocomplex as claimed in Claim 1 which is soluble in aqueous media.
3. A heterocomplex as claimed in Claim 1 which is insoluble in aqueous media.
4. A heterocomplex as claimed in any preceding claim in which the immunoglobulin has antigenic specificity against a particular virus.
5. An interferon-immunoglobulin cross-linked heterocomplex substantially as hereinbefore described.
6. A pharmaceutical preparation comprising a heterocomplex as claimed in any one preceding claim together with a pharmaceutically acceptable carrier.

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